XXI. SOME OBSERVATIONS ON YEAST GROWTH.

By ARTHUR SLATOR.

(Received June 8th, 1918.)

It has been shown for many growths of micro-organisms that the logarithmic law of increase holds good so long as growth takes place unrestrictedly in the nutrient medium. If N is the number per cc. at time t then $\frac{dN}{dt}/N$ is constant during this period of growth. This value $\frac{dN}{dt}/N$, which we may call Z , is the rate of growth per cell or the constant of growth and we have the equation $Z = a$, a constant¹.

The generation-time (g.t.) = $\frac{\log 2}{Z}$.

When an old culture of bacteria or yeast is seeded into a fresh nutrient medium the initial development takes place slowly. During this lag-phase in growth Z is initially small, it increases with the time and finally reaches the value of unrestricted growth.

It has been shown by Ledingham and Penfold [1914] that the growth of *B. coli* during this period of lag follows a definite law. On calculating Z from their equation² we find

$$Z = at^b,$$

where a and b are constants in each experiment but vary in the different experiments with different seedings. In eight experiments b has the values 0.88, 0.77, 0.56, 0.56, 0.97, 0.74, 1.01, 1.7. It has been further pointed out

¹ This logarithmic constant occurs in many branches of science and there are many ways of expressing it. The "constant" of a unimolecular chemical reaction, the period of "half-change" of a radioactive substance, the "birth rate" of a population, the "generation-time" in the growth of micro-organisms, "compound interest" on a sum of money, are some of the many examples which can be quoted. For a general method of recording such "velocity-constants" the inverse of the "generation-time" or the inverse of the period of "half-change" has much to recommend it and is in some respects simpler than that suggested by J. W. McBain [1917]. In this paper the usual method of giving velocity-constants is employed, but generation-times are also given.

² Ledingham and Penfold's equation reads $X^n = k \log Y$ where X is the time, and Y the number of bacilli (seeding = 1), k and n being constants. If the equation is put in the form $X^n K^n = \log Y^n$, K the new constant keeps the same value for the whole series of experiments.

Differentiating the equation we get

$$\frac{dY}{dX} / Y = \frac{K^n X^{n-1}}{0.434} = \frac{K}{0.434} (KX)^{n-1}.$$

Substituting Z for $\frac{dY}{dX} / Y$, a for $\frac{K}{0.434}$, t for KX , and b for $n - 1$, we have $Z = at^b$.

[Slator, 1917] that this equation can be slightly simplified. If a suitable unit of time be chosen the constant a in the equation $Z = at^b$ remains constant for the whole series of experiments. This equation $Z = at^b$ is not usual in chemical dynamics and it is of some interest to know whether it has any biological significance. Some interesting facts relating to growth of plants and animals are discussed by D'Arcy W. Thompson [1917, Chapter III]. The regenerative growth of tadpoles' tails seems to follow a law similar to that given above. Examples of rhythmic growth are also given.

It was in the hope of finding another example of this law that the lag-phase in the growth of yeast was investigated. It was found however that this period of growth is short and does not admit of measurements such as Ledingham and Penfold have carried out. The results are described below. An account of some further experiments on yeast growth is also included.

Method of measuring the rate of growth.

Direct observation of the growth of yeast cells under the microscope was found to be most suitable for the purpose of measuring the lag-phase of growth. A hollow slide was constructed through which water at any desired temperature could be circulated. By means of such a "warm slide" the medium in which the yeast cells were growing was kept at a desired constant temperature and a number of yeast cells were continually under observation under the microscope. Other methods of keeping the slide at a constant temperature would no doubt be effective. It is evidently of some convenience to deal with a rapidly growing yeast and most of the experiments were carried out with a baker's yeast growing at 30° in malt-wort of sp. gr. 1.040.

Baker's yeast growing unrestrictedly at 30°.

Such growth was observed to take place in the following manner. A bud appears and grows steadily for about $\frac{3}{4}$ hr when it has developed to about the same size as the mother cell. For another $\frac{1}{2}$ hr no appreciable change is to be noticed. Both cells then bud usually almost simultaneously and the process of growth is then repeated. By counting the cells at various intervals of time fair constants of growth can be obtained. In the following table t = time in hours, $N + n$ = the number of cells at time t , the initial number being N . K is calculated according to the equation

$$0.434 K = \frac{1}{t} \log \frac{N+n}{N}$$

Experiments 1 and 2 are single colony growths, in Exps. 3 and 4 groups of four colonies were counted.

TABLE I.

1	t	0	$1\frac{1}{2}$	$2\frac{1}{2}$	4	
	$N+n$	$4\frac{1}{2}$	10	16	35	
	0.434K	—	0.248	0.230	0.229	Average = 0.236
2		Repeat exp. gave				„ = 0.244
3	t	0	1	$1\frac{1}{2}$	3	
	$N+n$	$9\frac{1}{2}$	17	$28\frac{1}{2}$	57	
	0.434K	—	0.241	0.264	0.256	„ = 0.254
4		Repeat exp. gave				„ = 0.248

Average of the four = 0.246, g.t. = 1.22 hrs = 73 mins.

Another method of measuring this generation-time gave good results. It is carried out in the following manner. A yeast cell is observed to bud and the time t_1 noted. The bud grows and eventually two new buds appear, one on each cell usually within a few minutes of each other (Fig. 1). The mean time t_2 is observed. The difference $t_2 - t_1$ is the time of a complete generation. The method is suitable for estimating the g.t. of individual cells. Twelve cells watched in this way under the microscope gave values 78, 66, 76, 70, 79, 65, 81, 72, 72, 70, 72, 73 the average being 73 mins. The g.t.s of individual cells vary therefore up to 8 mins on each side of the average.

TABLE II.

Cell number	Time of appearance hrs mins	Average hrs mins	Time when fully grown hrs mins
2	0 0	0 0	0 45
3	1 18		
4	1 16	1 17	2 0
5	2 33		
6	2 33		
7	2 33		
8	2 33	2 33	3 15
9	3 49		
10	3 50		
11	3 52		
12	3 38		
13	3 45		
14	3 48		
15	3 52		
16	3 38	3 47	

Generation-times = 77, 76, 74, average 76 mins.

The following case of a single cell growing to sixteen cells is of interest. The time at which each cell appeared as a bud was noted and each successive average g.t. obtained from the time differences. In the table the number of each cell corresponds to that given in the diagram (Fig. 2).

The lag-phase.

If an old culture of yeast is seeded into wort an hour to two hours elapses before visible growth takes place. It is then found that growth proceeds at the maximum rate.

A five days old culture of yeast was seeded into fresh wort and the G.T. of thirteen cells estimated as soon as buds appeared. They gave values 81, 89, 80, 78, 77, 72, 85, 74, 88, 78, 76, 81, 70 mins; average 81 mins, a value only slightly greater than the minimum G.T. When older cultures were used similar results were obtained but more dead cells were present.

Fig. 1. Generation-time = $t_2 - t_1$.

Fig. 2. Diagram of yeast growth.

Individual cells show considerable variation in the time they remain quiescent before budding. Thirty-seven cells kept under observation gave the following results:

During the first 60 mins. 0 budded			from 91 to 95 mins. 2 budded		
from 60 to 65	6		96	100	4
66 70	7		101	105	0
71 75	4		106	110	1
76 80	8		111	115	1
81 85	1		116	120	1
86 90	1		121	125	1

The lag-phase in the growth of a yeast cell consists therefore of a quiescent stage of one or two hours varying with the individual cells and then growth at the normal unrestricted rate takes place.

Yeast growth proceeds in alternate periods of growth and rest and the lag is apparently little more than a prolonged period of rest. Many attempts have been made to deduce the equation $Z = at^b$ from such data but without success.

If it were possible to follow the growth of *B. coli* under the microscope and compare it with the results obtained by plating, an explanation of Ledingham and Penfold's equation might be forthcoming. It is worth noting that the death of some of the cells may take place during such measurements. Thus Penfold [1914, Table X, p. 230] records an initial decrease in the number of bacilli in some cases; that is a certain number of the original seeding die in the nutrient medium but grow if gelatin is present. The presence of a poison which diffuses slowly might account for the favouring influence of gelatin.

The growth of spores.

It was thought possible that the development of yeast spores might give a longer period of lag than is the case with quiescent yeast cells. Experiments were therefore made with a wild yeast which spores abundantly. Measurements of the G.T. of the yeast growing in malt-wort at 30° gave values 91, 82, 84, 81, 92, 95 mins, average 88 mins. When spores are kept under observation on the warm slide the general characteristics of spore growth are observed; the long period of rest (often 24 hrs) before visible development, the bursting of the cell walls and the budding of the spores. This wild yeast developed at first small round cells which grew in a mass and could not be counted. After about the second generation the normal larger cells developed, the rate of growth of which was easily estimated. Three cells gave values 94, 85, 88 mins, that is the maximum rate of growth is already attained. There is therefore little or no lag when once the spores have started developing.

Influence of carbon dioxide.

To obtain the maximum rate of yeast growth (minimum G.T.) it is essential that there should be no appreciable accumulation of carbon dioxide in the nutrient medium. This is the case if the number of yeast cells does not exceed a few hundred thousand per cc. That carbon dioxide has an appreciable retarding influence is easily demonstrated. If the experiments are carried out with wort saturated with carbon dioxide as the nutrient medium, a number of the cells are observed to die and those which survive grow at about half the maximum rate. Yeast which has been growing some time in the presence of carbon dioxide no longer exhibits the dying off effect and grows rather faster, but in no case was the maximum rate of growth observed. The effect of carbon dioxide on yeast growth is well known and seems to be general. Air which has a large influence in the later stages of yeast growth has no influence when dealing with these small concentrations of yeast, for rates of growth measured under these conditions in wort saturated with air and in wort from which the air had been evacuated gave the same result.

Yeast growth at high temperatures.

If rates of growth at different temperatures are plotted against the temperature a characteristic curve is obtained. Below a certain low temperature and above a certain high one no growth takes place. Between the two the rate of growth varies greatly. As the temperature rises the rate increases and finally attains a maximum. A further increase in the temperature causes a rapid

decrease in the rate which finally becomes zero. In an investigation of the influence of temperature on the rate of growth of *B. delbrücki* it was observed that the rate was about halved when the temperature was raised from 59° to 52.5° [Slator, 1916]. The rate however remained constant at the higher temperature. It is of interest to know whether the lower rate is due to cell destruction or to slower rates of growth of the individual cells. By observing growth on the "warm slide" this point can be settled as far as yeast cells are concerned. The baker's yeast used in these experiments gave an average G.T. of 63 mins at 39° to 40°. At 41° retardation in growth takes place and some of the cells also die. Observations of a group of four cells growing at 41° gave the following result, one cell died before budding and the other three gave G.T. 67, 77, 85 mins. Other groups of cells showed similar results—some dying and some growing. At these high temperatures therefore some of the cells grow at about the maximum rate, some at a considerably lower rate and some die.

The rate of spore production in yeasts is influenced by temperature in a similar manner to growth. These rates are usually measured in the following way. An actively growing culture is spread over a moist gypsum block and kept at a constant temperature. An examination is made periodically and the time of the first appearance of spores is noted. The times observed are therefore the minimum for spore production at the given temperature. If one constructs curves plotting temperature against the inverse of these times the similarity to temperature-rate of growth curves is very noticeable. Here again we have the upper and lower limiting temperatures, a maximum rate, and a part of the curve where the temperature coefficient is less than one. We are dealing here only with the few cells which form spores in the shortest possible time and the slower rates at high temperature cannot be explained by cell destruction. Probably both with spore production and with growth at temperatures above that of maximum rate partial destruction of some substance affecting the rate takes place but is not sufficient to stop development entirely.

In the case of enzyme action where a similar maximum occurs temperature coefficients less than one are usually attributed entirely to destruction of the enzyme.

Other uses of the "warm slide."

This method of investigating yeast growth can be conveniently used in all cases when rapid growth takes place. It is also of use in determining the initial effect of poisons on growth, thus a dying off effect or a lag-phase is soon detected by this means whilst they may be missed entirely if rates of growth are estimated by ordinary counting methods.

Use of the "warm slide" was made to throw some light on the effect of yeast growth on diastatic action. The experiments showed no new results but they suggested a point which is worth mentioning. Diastase (malt extract) has a slow solvent action on malt starch at ordinary temperatures and this reaction is accelerated if yeast is present. G. H. Morris [1901] showed that the products of reaction of diastase on starch have no influence on the rate of the reaction and it is unlikely therefore that removal of the reaction products by fermentation would account for the acceleration. The action of diastase on starch is accelerated by carbon dioxide [for references see Euler, 1912, p. 158] and no doubt the carbon dioxide evolved by the yeast influences the reaction. Whether this effect accounts for the acceleration entirely requires other experiments to prove but it certainly has to be taken into consideration.

The influence of oxygen on yeast growth.

The action of air (oxygen) on yeast growth has attracted attention from the time of Pasteur onwards and rightly so, for oxygen plays an important part in yeast growth. One of the most striking ways of showing that air is necessary to yeast growth is to attempt to grow "giant colonies" of yeast on wort gelatin in an evacuated vessel. Growth is very poor and liquefaction of the gelatin soon takes place under these anaerobic conditions.

Within the last few years H. T. Brown [1909, 1914] has more thoroughly investigated this influence of oxygen. In agreement with A. J. Brown [1905] he shows that when yeast develops in malt-wort, growth is retarded and finally ceases at an earlier stage than one would expect taking into consideration the food requirements of the yeast and the retarding influence of alcohol and carbon dioxide on growth. He concludes that lack of oxygen is the main factor which causes this retardation. The experiments described here are in accordance with this conclusion. He further finds that very considerable yeast growth takes place under strictly anaerobic conditions. This admits of explanation on the assumption that there is a certain amount of chemically combined oxygen in the wort which is available for yeast growth. Subsequently [1914] H. T. Brown discards this explanation and considers that the yeast used for seeding carries oxygen with it in the form of an "oxygen charge" and that growth is determined by this "charge."

The theory gives a ready explanation of the approximately rectilinear curves of growth to which he draws attention. The alternative explanations depend on whether retardation in growth is due to change in the medium (lack

of combined oxygen) or to change in condition of the yeast (continual splitting of the "oxygen charge").

TABLE III.

<i>Aerobic growth at 20°</i>			<i>Anaerobic growth at 20°</i>		
<i>t</i> (hrs)	<i>N + n</i>	0.434 <i>K</i>	<i>t</i>	<i>N + n</i>	0.434 <i>K</i>
0	11,500	—	0	11,500	—
22.5	630,000	0.077	42 hrs	322,000	0.034
42	11,200,000	0.071			
0	226	—	0	226	—
42½	175,000	0.068	4½ days	266,000	0.027
47	441,000	0.070	5 "	1,440,000	0.032
0	4.4	—	0	4.4	—
66½	365,000	0.074	9 "	7,000,000	0.029
		0.072			0.031
G.T. = 4.2 hrs			G.T. = 9.7 hrs		

Estimations of the rate of growth with very small seedings were made to decide between these two alternatives. The experiments described below show fairly conclusively that there is in malt-wort combined oxygen available for yeast growth. Rates of growth were estimated by yeast counts according to Method 1 previously described [Slator, 1913], a Burton brewery yeast being used for the purpose. It grew at a rate corresponding to a G.T. of 3 hrs at 20° in malt-wort of sp. gr. 1.040, and the same results were obtained whether the experiments were carried out in the presence of air or in evacuated tubes. All attempts to obtain lower rates by drastically excluding air were unsuccessful. Efforts were then made to find a medium in which growth is influenced by air. Half-fermented wort in which yeast growth had stopped was filtered and partly evaporated in an open dish to free it from alcohol and growth in this medium tested. It was found however that yeast grew in this medium both anaerobically and aerobically at the same rate. Wort of sp. gr. 1.060 was then taken, fermented until it contained about 2 % of alcohol and filtered out of contact with air. It was then evaporated in an evacuated distilling flask to bring it back to about its original gravity of 1.060. In this medium yeast behaved differently. Rates of growth under anaerobic conditions were much smaller than under aerobic conditions. Table III gives the results of measurements of rates of growth with different seedings; in one case the experiments were carried out with free access of air, in the other under anaerobic conditions in a number of evacuated tubes. A G.T. of 4.2 hrs was observed when air was present and a G.T. of 9.7 hrs when air was absent. The seeding in each case

was made with yeast which was grown under anaerobic conditions. The presence of air was found to favour the growth of smaller cells than were produced when air was absent, but as the increase over the period of growth is great the difference in the size of the cells would hardly affect the velocity-constants.

There is no doubt that partly fermented wort differs from the original wort in that combined oxygen available for yeast growth is present in the one case and very much reduced in the other. The effect of oxygen on yeast growth deserves more attention than is given in this short note, but apparently dissolved oxygen (which is rapidly absorbed by the yeast) and oxygen chemically combined in the wort are both available for yeast growth. The necessary amounts are small; thus Brown [1914] estimates that 1.7 cc. of oxygen are used up in the growth of 10^{10} cells (about 2.5 g. of pressed yeast).

SUMMARY AND DISCUSSION OF RESULTS.

A method of measuring rates of growth of yeast by direct observation under the microscope is described. The G.T. of individual cells of a baker's yeast growing in malt-wort at 30° varies up to 10 % on each side of the average value of 73 mins.

Measurements of the lag-phase in the growth of yeast cells show that old yeast cells remain quiescent for some time when introduced into a fresh medium, the time of quiescence varying with different cells. They then start growing at the normal unrestricted rate. Yeast growth proceeds in alternate periods of rest and growth, and lag is apparently little more than a prolonged period of rest.

Little or no lag is observed in yeast cells grown from spores, when once development has started.

The retarding influence of carbon dioxide is readily observed by this method of measuring growth.

At a temperature above that of maximum rate of growth some cells grow at about the maximum rate, some at lower rates and some die.

The influence of oxygen on yeast growth has been tested by measuring generation-times under anaerobic and aerobic conditions. The results confirm previous conclusions that air is necessary for yeast growth, and that oxygen chemically combined in the wort as well as dissolved oxygen which is rapidly absorbed by the yeast is available for yeast growth. A method of preparing a medium in which yeast growth is sensitive to air is described.

If yeast is seeded into malt-wort the following stages of growth may be observed: the lag-phase or period of quiescence, the logarithmic phase or period of unrestricted growth, retardation in growth due to carbon dioxide, retardation in growth due to failure of oxygen. The latter factor is the main one which finally stops growth. The accumulation of alcohol and lack of necessary nutrients seldom come into consideration up to this stage. When fermentation is over air may have access and cause a regrowth of the yeast, the alcohol and possibly lack of nutrients such as assimilable sugar are then the main retarding factors.

It depends on conditions of seeding, aeration, temperature, etc., how far these different stages of growth become prominent, overlap or disappear. Thus if the seeding consists of actively growing yeast the lag-phase disappears, if the seeding amounts to a few million per cc. retardation starts at once and there is no logarithmic period, if the seeding is very great lag in growth and rapid accumulation of retarding influences may prevent growth entirely. The problem of the final yeast crop is largely a matter of rates of growth with failing supplies especially of oxygen.

Thanks to the work of A. J. Brown, A. L. Stern [1899, 1901], H. T. Brown and others it should not be difficult to determine rates of growth with definite small concentrations of known essential nutrients and possibly put the problem of yeast crops on a sound mathematical basis.

In this and in previous papers [1913, 1916] several methods of measuring the logarithmic constant of growth and the generation-time of micro-organisms have been described, and attempts have been made to show the importance of these measurements in an investigation of the growth of micro-organisms and the chemical action brought about by them. The figures obtained are not merely interesting mathematical curiosities. They represent accurate measurements of growth under conditions where *the medium remains practically unaltered*, and they are of considerable value in solving problems connected with the growth of micro-organisms. It may be said that yeast (for instance) for practical purposes never grows under such conditions and that measurements under "normal" conditions are of more value. The argument is only partly correct for one can investigate phases of retarded growth (such as when yeast growth is retarded by lack of oxygen) by this method, and in general it is better to study each part of the puzzle under the simplest possible conditions and then attempt to fit the pieces together into a harmonious whole. The method is in fact that of investigating growths of micro-organisms and the chemical actions brought about by them in the same manner as one would investigate the

kinetics of a purely chemical reaction. One has every confidence that the method will justify itself in throwing fresh light on these problems. Much of the work carried out in connection with the fermentation industries especially relating to growths of yeast, acetic acid bacteria, lactic acid bacteria, etc., is sufficiently far advanced to be investigated from this point of view and such measurements would be well worth making. The field of investigation is by no means limited to these organisms but extends to all branches of science in which micro-organisms play a part.

In conclusion I desire to express my thanks to Dr A. L. Stern, with whom I have had the advantage of discussing the subject matter of the present paper.

REFERENCES.

- Brown, A. J. (1905), *J. Chem. Soc.* **87**, 1395.
Brown, H. T. (1909), *J. Inst. Brew.* **15**, 169.
— (1914), *Annals of Botany*, **28**, 197.
Euler, H. (1912), *General Chemistry of the Enzymes*, translated by T. H. Pope.
Ledingham, J. C. G. and Penfold, W. J. (1914), *J. Hygiene*, **14**, 242.
McBain, J. W. (1917), *Trans. Faraday Soc.* **13**.
Morris, G. H. (1901), *J. Chem. Soc.* **79**, 1085.
Penfold, W. J. (1914), *J. Hygiene*, **14**, 215.
Slator, A. (1913), *Biochem. J.* **7**, 197.
— (1916), *J. Chem. Soc.* **109**, 2.
— (1917), *J. Hygiene*, **16**, 100.
Stern, A. L. (1899), *J. Chem. Soc.* **75**, 201.
— (1901), *J. Chem. Soc.* **79**, 943.
Thompson, D'Arcy W. (1917), *Growth and Form*.